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Production and characterization of a monoclonal antibody that cross-reacts with the mycotoxins nivalenol and 4-deoxynivalenol

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Abstract
Nivalenol is a mycotoxin produced by certain fungi that are pathogenic to important cereal crops, in particular maize, wheat, and barley. This toxin, 3α,4β,7α,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one, is found worldwide and is closely related to 4-deoxynivalenol (DON or vomitoxin), a mycotoxin associated with outbreaks of *Fusarium* head blight in North America. The literature on the toxicity of nivalenol suggests it is similar, if not more toxic, than DON. Despite the development of rapid immunologically based assays for detecting DON, such assays have not existed for detecting nivalenol without chemical modification of the analyte. This paper describes the development of a monoclonal antibody using a nivalenol–glycine protein conjugate. The monoclonal antibody was most specific for an acetylated form of DON (3-Ac-DON), but it exhibited sensitivity and cross-reactivity that were useful for detecting nivalenol and DON at relevant levels without the need to modify either toxin chemically. In an competitive indirect ELISA format, the concentrations of toxins able to inhibit colour development by 50% (IC_{50}) were 1.7, 15.8, 27.5, 68.9, and 1740 ng ml^{-1} for the mycotoxins 3-Ac-DON, DON, nivalenol, 15-Ac-DON, and fusarenon-X, respectively. The antibody was also used to develop a competitive direct ELISA for DON and nivalenol, with IC_{50}'s of 16.5 ng ml^{-1} (DON) and 33.4 ng ml^{-1} (nivalenol). These assays are capable of detecting both DON and nivalenol simultaneously, a property that may be useful in regions where these toxins co-occur or in formats, such as immunoaffinity columns, where co-isolation of both toxins is desirable.

Keywords: *Nivalenol, trichothecene, mycotoxin, antibody, immunoassay*

Introduction

Certain species of *Fusaria*, in particular *F. graminearum* and *F. culmorum*, cause several diseases in economically important grains, including head blight (scab) in wheat and ear rot in maize. Often, the infested grain contains mycotoxins, in particular 4-deoxynivalenol (DON) and structurally related trichothecenes. Nivalenol (NIV), 12,13-epoxy-3α,4β,7α,15-tetrahydroxy-trichothec-9-en-8-one, is structurally similar to DON, the difference being a hydroxyl group at the C4 position (Figure 1). While *Fusarium* head blight and maize ear rot occur worldwide, there are distinct geographical differences in the toxins produced. DON is the predominant trichothecene produced by strains of *F. graminearum* found in the USA. However, there is a second chemotype of *F. graminearum* that produces NIV (Pronk et al. 2002). In Asia and Europe, both chemotypes, and therefore both toxins, may be present (Tanaka et al. 1985; 1988 Kim et al. 1993; Yoshizawa and Jin 1995).

NIV and DON are both capable of inhibiting protein synthesis and inhibiting DNA and RNA synthesis. Because of this, they are especially toxic to rapidly dividing tissues. The immune system is a primary target for trichothecene toxicity. For a comprehensive summary of the toxicology and occurrence of NIV and related trichothecenes, see Pronk et al. (2002).

The US Food and Drug Administration (FDA) has set advisory levels for DON ranging from 1 μg g^{-1} (ppm) for finished wheat products destined for human consumption up to 5 μg g^{-1} in the diet of ruminating beef cattle, feedlot cattle, and chickens. The European Commission's Scientific Committee

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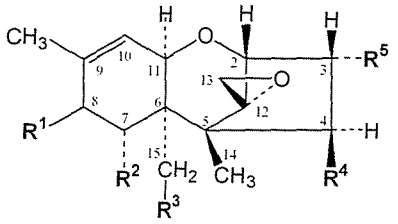

Common name	R^1	R^2	R^3	R^4	R^5
Nivalenol	=O	OH	OH	OH	OH
Deoxynivalenol	=O	OH	OH	H	OH
3-Acetyl-deoxynivalenol	=O	OH	OH	H	OAc
15-Acetyl-deoxynivalenol	=O	OH	OAc	H	OH
Triacetyl-deoxynivalenol	=O	OAc	OAc	H	OAc
Fusarenon-X	=O	OH	OH	OAc	OH
Trichothecin	=O	H	H	X	H
8-Hydoxy-isotrichodermin	OH	H	H	H	OAc
T-2 tetraol	OH	H	OH	OH	OH
Isotrichodermin	H_2	H	H	H	OAc
Diacetoxyscirpenol	H_2	H	OAc	OAc	OH
Verrucarol	H_2	H	OH	OH	H
Neosolaniol	OAc	H	OAc	OAc	OH
T-2 toxin	ISV	H	OAc	OAc	OH
T-2 triol	ISV	H	OH	OH	OH
HT-2 toxin	ISV	H	OAc	OH	OH

OAc, ISV and X are $-OCOCH_3$, $-OCOCH_2CH(CH_3)_2$, and $-OCOCH=CHCH_3$, respectively.

Figure 1. Structure of NIV and related trichothecene mycotoxins.

on Food (SCF) established a tolerable daily intake (TDI) of 1 $\mu g\,kg^{-1}$ body weight day^{-1} for exposure to DON and a temporary TDI of 0.7 $\mu g\,kg^{-1}$ body weight day^{-1} for exposure to NIV (Pronk et al. 2002; Scientific Committee on Food 2002).

Two aspects make monitoring of NIV of interest, namely the increase in global trade and the potential for worldwide distribution of NIV-producing strains of *F. graminearum*. The majority of methods for the analysis of NIV in foods use chromatographic approaches such as gas chromatography (GC) or high-performance liquid chromatography (HPLC). Analytical methods of trichothecenes have been reviewed (Langseth and Rundberget 1998; Krska et al. 2001; Schneider et al. 2004). For the chromatographic methods, NIV has been extracted from grains using a variety of solvents, most notably aqueous mixtures of acetonitrile and methanol. Acetonitrile–water in proportions of 84 : 16 (v/v) is most commonly used, especially in combination with charcoal–alumina solid-phase extraction (SPE) clean-up columns. Charcoal–alumina, florisil, and silica columns have all been used for the clean-up or isolation of NIV (Yoshizawa 2001). Immunoaffinity columns have also been used for the isolation of DON, but not NIV, before analysis.

GC combined with flame ionization detection or electron capture detection (GC-ECD) has been used extensively for the analysis of DON and NIV. Most GC-ECD methods involve derivatization of the hydroxyl groups of NIV to increase the volatility of the mycotoxin and to enhance the sensitivity. The derivatization is generally accomplished by forming the corresponding trimethylsilyl (TMS) ethers or perfluoroacyl esters. The TMS or perfluoroacyl derivatives are also amenable to GC with mass spectrometric detection (GC-MS; Tanaka et al. 2000; Nielsen and Thrane 2001).

NIV, like DON, has an absorption band in the ultraviolet region that permits the use of HPLC with ultraviolet light for detection (Walker and Meier 1998). To improve sensitivity, trichothecenes have also been labelled with fluorescent tags. Pre-column derivatization reagents reported include anthracene 9-carbonyl chloride, coumarin 3-carbonyl chloride, and 1-anthroyl nitrile (Visconti et al. 2005). HPLC combined with either atmospheric pressure chemical ionization (APCI) or electrospray MS detection is another alternative that is becoming more widely used (Berthiller et al. 2005; Biselli et al. 2005). The chromatographic methods offer excellent opportunities for detecting and quantitating NIV. However,

most are not suitable as rapid screening assays for this toxin.

Although NIV has been detected using antibody-based methods, highly specific antibodies for NIV have not been reported. The antibody-based methods have measured NIV in one of two ways: by derivatizing the toxin before detection, or by using antibodies specific for other trichothecenes but which cross-react with NIV. Antibodies have been developed using congeners such as 3,15-diacetyl-deoxynivalenol, 4,15-diacetyl-nivalenol, 3,7,15-triacetyl-deoxynivalenol (Tri-Ac-DON), 3,4,15-triacetyl-nivalenol, and 3,4,7,15-tetracetyl-nivalenol (Zhang et al. 1986; Wang and Chu 1991; Abouzied et al. 1993; Kohno et al. 2003). Several have been used to detect DON or NIV in foods or fungal cultures following acetylation (Xu et al. 1988; Teshima et al. 1990; Schmitt et al. 1996; Yoshizawa et al. 2004). While in all cases cross-reactivity with NIV was poor, the assays were able to detect the acetylated NIV (Zhang et al. 1986; Teshima et al. 1990; Wang and Chu 1991; Abouzied et al. 1993; Kohno et al. 2003; Yoshizawa et al. 2004).

An alternative approach to derivatization of the samples would be to use antibodies that cross-react significantly with NIV itself. Two trichothecenes with similar structures are DON and fusarenon-X (FX) (Figure 1). Several groups have used these toxins for making trichothecene antibodies (Märtlbauer et al. 1989; Mills et al. 1990; Usleber et al. 1991; Nicol et al. 1993; Niessen et al. 1993; Sinha et al. 1995; Lee et al. 1997; Maragos and McCormick 2000; Schneider et al. 2000). However, a monoclonal antibody (Mab) sensitive enough for screening of NIV in foods has not been reported.

As a result, immunoassays for NIV have been restricted to those incorporating acetylation steps. Previously, the present authors' group has reported the development of three Mabs that bind DON. However, those antibodies did not cross-react significantly with NIV in either chemical ionization competitive indirect ELISA (CI-ELISA), fluorescence polarization, or biosensor formats (Maragos and McCormick 2000; Maragos et al. 2002; Tüdos et al. 2003). The present paper describes the development of a Mab that is most specific for 3-Ac-DON, but which exhibits sensitivity and cross-reactivity that are useful for detecting NIV and DON at relevant levels.

Materials and methods

Reagents

Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead, Boston, MA, USA) was used in the preparation of all reagents. All solvents were HPLC grade. The NIV used to produce chicken egg albumin (ovalbumin, OVA), bovine serum albumin (BSA) and horseradish peroxidase conjugates (HRP) was prepared at the National Institute of Health Sciences (Tokyo, Japan). Triacetyl-DON, isotrichodermin, 8-hydroxy-isotrichodermin, and sambucinol were provided by Dr Susan McCormick (USDA-ARS-NCAUR, Peoria, IL, USA). DON, 3-acetyl-deoxynivalenol (3-Ac-DON), 15-acetyl-deoxynivalenol (15-Ac-DON), fusarenon-X (FX), diacetoxyscirpenol (DAS), trichothecin, T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, neosolaniol, roridin A, verrucarin A, and verrucarol were obtained from Sigma Chemical (St Louis, MO, USA). Other reagents purchased from Sigma Chemicals included OVA, BSA, HRP, and polyvinyl alcohol. 1,1′-Carbonyldiimidazole (CDI) and 1[3-(dimethylamino)-propyl]-3-ethyl-carbodiimide hydrochloride (EDC) were purchased from Aldrich (Milwaukee, WI, USA). *O*-methylglycine (OMG) was purchased from Bachem (King of Prussia, PA, USA). Peroxidase-conjugated goat anti-mouse immunoglobin-G (IgG) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other chemicals and solvents were reagent grade or better and purchased from major suppliers.

Preparation of NIV–Gly adducts

The hydroxyl groups of NIV were linked to the primary amine of *O*-methylglycine (OMG) using CDI similar to that reported for linking DON to proteins (Maragos and McCormick 2000). The reaction products, a mixture containing OMG attached through one or more of the hydroxyl groups of NIV, were partially purified over a reverse-phase SPE column. The NIV–OMG products were then separated by reverse-phase HPLC and the methyl ester was removed with alkali. The NIV–Gly products were further purified using reverse-phase SPE and their presence was confirmed by liquid chromatography (LC)-electrospray mass spectrometry (LC/ESI/MS).

Briefly, 600 mg CDI was added to 60 mg NIV in 1.8 ml dimethylsulfoxide, sealed tightly and mixed for 1 h at ambient temperature, after which 0.06 ml water were added, followed by OMG solution (36 mg in a 2.4 ml sodium carbonate buffer, pH 9.4). This mixture was held at ambient temperature for 23 h with stirring, then neutralized with 1 N hydrochloric acid. The mixture was diluted with water and the entire volume passed through a C18 SPE column (Varian Mega Bond Elut, 5 g sorbent mass; Varian, Walnut Creek, CA, USA). The SPE column was washed with water and 5% aqueous

methanol before eluting the crude NIV–OMG with 50% aqueous methanol. The eluate was taken to dryness, solubilized in minimal methanol/water and the entire volume applied to a reverse-phase HPLC. The pump was a model P2000 (Thermo Separations, San Jose, CA, USA) equipped with a 1 ml manual injector loop (Model 9125 injector; Rheodyne), a guard column (Prep Nova-Pak HR C18, 6 µm, 60 Å, 25 mm i.d. × 10 mm; Waters, Milford, MA, USA), and a Waters Prep-Pak cartridge (Nova-Pak HR C18, 6 µ, 60 Å, 25 mm i.d. × 100 mm). The mobile phase was a stepwise gradient of methanol and water, beginning with 10% methanol for 10 min 20% methanol for 10 min, and 30% methanol for 10 min, followed by a linear gradient from 30 to 50% methanol over 20 min (a total gradient time of 50 min). Fractions were collected every 2 min, pooled where appropriate, and concentrated to dryness. Dried fractions believed to correspond to the mono-substituted (NIV–OMG) and di-substituted [NIV–$(OMG)_2$] derivatives were solubilized in minimal methanol and treated with 0.02 M sodium borate (pH 9.5) to remove the methyl ester(s). After neutralizing with 1 N hydrochloric acid, the materials were applied to a C18 SPE column. The column was washed with water and the putative monolabelled (NIV–Gly) or di-labelled [NIV–$(Gly)_2$] materials eluted with 25 ml of 50% aqueous methanol. These materials were taken to dryness and examined by HPLC-ESI-MS in order to confirm the presence of the desired products.

LC/ESI/MS of the NIV–Gly fraction

All analyses were conducted on a Thermo Finnigan LCQ DECA ion-trap mass spectrometer coupled to a Thermo Spectraphysics HPLC system. The HPLC system consisted of a P4000 quaternary pump and an AS3000 autosampler. Reverse-phase chromatography was done using a 0.3 mm i.d. × 150 mm MetaChem Inertsil 5 C_{18} column with a flow rate of 300 µl min^{-1}. A linear gradient going 5 to 90% methanol over 25 min was used to elute the analytes. The entire flow from the column was directed to the electrospray interface of the mass spectrometer. The mass spectrometer was operated in either MS or MS/MS mode. For the MS/MS experiments, alternating scans were used to acquire sequentially full-scan MS and full-scan product-ion MS/MS from parent ions of interest.

Preparation of NIV protein conjugates

Protein conjugates of NIV were synthesized by linking the carboxylic acid of the NIV–Gly with the primary amines of the proteins. The immunogen, a conjugate of NIV–Gly and OVA (NIV–Gly–OVA), was prepared using a water-soluble carbodiimide method. Briefly, 20 mg OVA was combined with 7.6 mg NIV–Gly and 150 mg EDC in 0.1 M MES (pH 5) and reacted for 3 h at ambient temperature, with stirring. The products (NIV–Gly–OVA) were dialysed sequentially against several changes of 0.1 M 2-morpholino-ethanesulfonic acid (MES), 0.01 M phosphate-buffered saline (PBS), and 0.1 M PBS at 4°C.

The test antigen, a conjugate of NIV–Gly with BSA (NIV–Gly–BSA), was prepared using a mixed anhydride procedure. Briefly, 10 mg of NIV–Gly was dissolved in minimal dimethylformamide and 0.015 ml triethylamine and 0.015 ml isobutylchoroformate were added. The mixture was held at −5°C for 15 min, after which BSA solution (22 mg in 1.5 ml 0.13 M aqueous sodium bicarbonate) was added. This solution was stirred and 0.06 ml of 0.1 N sodium hydroxide was added. The mixture was held at 4°C with stirring for 4 h, then 1 h at ambient temperature, before transferring to dialysis tubing. The NIV–Gly–BSA was dialysed sequentially against several changes of 0.01 M PBS, and 0.1 M PBS.

The protein concentrations of the conjugates were determined by assay with bicinchoninic acid (Pierce, Rockford, IL, USA). The conjugates were diluted to 2 mg ml^{-1} with 0.1 M PBS, then distributed as 0.25 ml portions and lyophilized. The lyophilized material was reconstituted with water before the immunization of mice.

Immunizations

All animal work and cell culture experiments were conducted at Harlan Bioproducts for Science (Madison, WI, USA). Ten female Balb/C mice were initially immunized by the injection of 100 µg NIV–Gly–OVA per animal using the same procedures as described previously for the production of DON antibodies using DON–OVA conjugates (Maragos and McCormick 2000).

Screening for NIV-specific antibodies by CI-ELISA

A (CI)-ELISA was developed for the screening of mouse sera and culture supernatants for the presence of antibodies. For screening assays, 0.1 ml of NIV–Gly–BSA conjugate, 100 ng ml^{-1} in 0.05 M sodium phosphate buffer (pH 7), was added to wells of polystyrene microtitre plates and allowed to attach overnight at 4°C. In later assays, for comparison of different clones and for cross-reactivity studies, the level of NIV–Gly–BSA coated was further reduced to 10 ng ml^{-1}. After washing the coated plate twice with 0.32 ml Tween-PBS (0.02% Tween-20 in 0.01 M PBS pH 7), 0.32 ml of polyvinyl alcohol PBS (1% PVA in 0.01 M PBS) were added and allowed to

incubate at ambient temperature for 1 h. During this incubation, test solutions were prepared. The test solutions consisted of 0.06 ml of toxin standard solutions (or PBS control) mixed with 0.06 ml of diluted serum (or culture fluid) diluted in OVA-PBS (1% w/v OVA in 0.1 M PBS) in the wells of a polypropylene microwell plate (Corning, Inc., Corning, NY, USA). The wells of the polystyrene (NIV–Gly–BSA-coated) plate were washed twice with Tween-PBS and 0.1 ml of test solution was transferred into each well. After incubation at ambient temperature for 30 min, the plate was washed three times and 0.1 ml of goat anti-mouse peroxidase conjugate (diluted 1 : 2000 in OVA-PBS) was added. The plate was incubated for 30 min at ambient temperature then washed four times before the addition of 0.1 ml of the substrate, *o*-phenylenediamine (OPD). The OPD solution was prepared by combining 0.02 ml of 30% H_2O_2 and 20 mg OPD in 50 ml of citrate-phosphate buffer (0.05 M citrate, 0.1 M phosphate, pH 5.0). After 5 min at ambient temperature, the reaction was stopped by the addition of 0.1 ml of 1 N hydrochloric acid. Colour development was determined by measuring the absorbance at 490 nm using a Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA).

Production and purification of Mab

Mice having sera with antibodies reactive to NIV were sacrificed and aseptically splenectomized. Spleenocytes were chemically fused with Balb/C non-immunoglobulin secreting (NS-1) myeloma cells using polyethylene glycol. Fused cells were plated in HAT (hypoxanthine, aminopterin, thymidine) selection media. After 11 days, HAT-resistant cultures were isolated and screened for anti-NIV activity by CI-ELISA. Positive cultures were subsequently cloned, expanded and frozen. A clone that screened positive was designated 1-6.2.6 and was expanded for ascites fluid production using established procedures (Hoogenraad et al. 1983). The ascites fluid was partially purified by ammonium sulfate precipitation using procedures described previously (Maragos and McCormick 2000).

Cross-reactivity with related trichothecenes

For cross-reactivity studies, stock solutions of 17 trichothecene mycotoxins were prepared in either acetonitrile or acetonitrile/water (1:1, v/v) depending upon the solubility characteristics of the toxin. Once in solution (generally at 1–2 mg ml^{-1}), the toxins were diluted further to obtain 500 µg ml^{-1} working stock solutions. The working stock solutions were used to prepare standards for analysis by diluting in PBS. The standards in PBS were then tested in the CI-ELISA, as described above. Many of the trichothecenes reacted poorly with the antibody and were consequently tested at very high concentrations. The greatest concentration tested, for the poorly reactive compounds, was 10 000 ng ml^{-1}. Concentrations above this level were not tested due the potential effects of residual acetonitrile upon the assay.

Effects of solvent upon assay performance

The effects of up to 20% aqueous acetonitrile or methanol were tested by preparing NIV standards over the range 0.1–2000 ng ml^{-1} in either PBS, 10% methanol in PBS, 10% acetonitrile in PBS, 20% methanol in PBS, or 20% acetonitrile in PBS. Standards in the indicated solution were tested by CI-ELISA, as described above.

Competitive direct ELISA for DON and NIV

The Mab 1-6.2.6 was also tested in a competitive direct ELISA (CD-ELISA) format. In this format, antibody was coated to the wells of microtitre plates and toxin (DON or NIV) was allowed to compete with a DON–HRP conjugate for binding. The Mab was coated at a 1:2000 dilution of the purified ascites, which corresponded with a protein concentration of 3.2 µg ml^{-1}. DON and NIV standards were prepared in PBS, as described above. The preparation of the DON–HRP conjugate and the CD-ELISA protocol were described previously for antibodies other than Mab 1-6.2.6 (Maragos and McCormick 2000).

Results and discussion

Preparation of NIV–Gly conjugates

NIV has four hydroxyl groups, at positions 3, 4, 7, and 15 (Figure 1). Three of these react readily with hydroxyl-specific reagents; the fourth (at position 7) is less reactive, perhaps because of the proximity of the carbonyl at position 8. The presence of multiple hydroxyl groups implies that multiple products may result when attempts are made to produce protein conjugates through them. A likely outcome of administering such mixtures to animals would be the production of many antibody variants, few with a high specificity for the desired toxin. For this reason, it was decided to modify NIV with a linker group (*o*-methylglycine), then partially to purify the NIV–OMG products before removing the methyl ester(s) to generate NIV–Gly products. The NIV–Gly products contained mono-substituted derivatives (NIV–Gly) and di-substituted derivatives [NIV–$(Gly)_2$]. The fraction containing the mono-substituted NIV–Gly was tested by LC-MS. Because the HPLC conditions incorporated ammonium

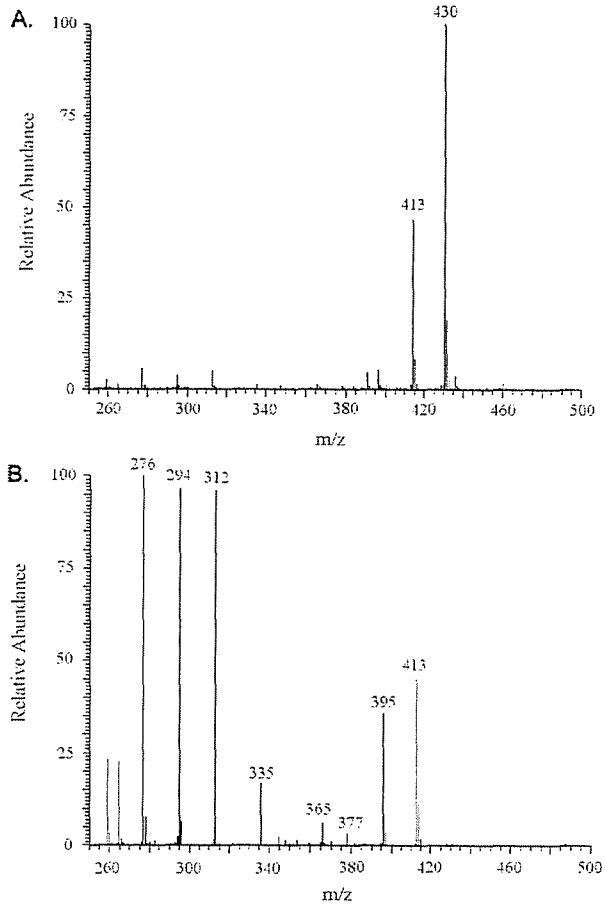

Figure 2. LC-MS spectrum of the NIV-Gly fraction used for preparing the protein conjugates. (A) Full mass spectrum. Major peaks correspond to the ammonium salt of NIV-Gly [M+NH4] + and NIV-Gly itself [M + H]+, with calculated molecular masses of 430.4 and 413.4 respectively. (B) MS-MS of the NIV-Gly, a fragment of which had a mass to charge ratio (m/z) of 312, corresponding to NIV (calculated mass 312.3).

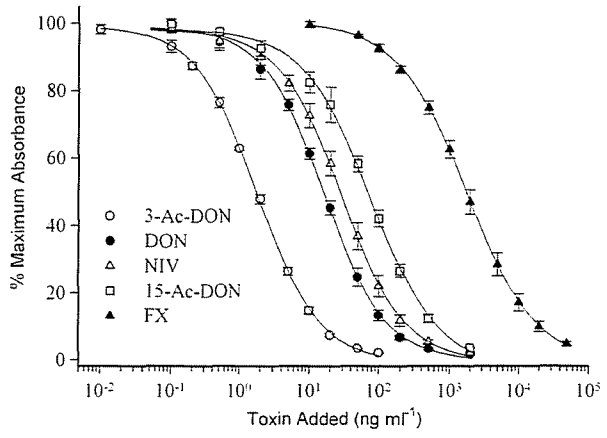

Figure 3. Response of 5 trichothecenes in the CI-ELISA with Mab 1-6.2.6. Curves are the fit of a logistic dose-response equation to 11 concentrations of toxin and a non-toxin control. Points shown represent the average ±1 standard deviation of data from 4 to 8 replicate plates, with quadruplicate replicates at each concentration on each plate.

acetate, the NIV–Gly was detected as both the ammonium salt and the acid (Figure 2).

The NIV–Gly was conjugated to two proteins (BSA, OVA). The NIV–Gly–OVA was administered to mice to generate an immune response, while the NIV–Gly–BSA was used as a test antigen in CI-ELISAs to screen sera for NIV antibodies. The approach was not particularly efficient, as only a single NIV-reactive antibody was able to be isolated from all of the screening, fusion, hybridoma production, and cloning steps. However, the resulting clone, designated 1-6.2.6, exhibited good activity toward the desired toxin, NIV.

Cross-reactivity of Mab 1-6.2.6 in the CI-ELISA format

The response of Mab 1-6.2.6 to five structurally related trichothecenes is shown in Figure 3. The antibody was most specific for 3-Ac-DON, followed by DON, NIV, 15-Ac-DON, and FX. The IC_{50}'s for these toxins in the CI-ELISA were 1.7, 15.8, 27.5, 68.9, and 1740 ng ml^{-1}, respectively. Setting the activity for 3-Ac-DON as 100%, the cross-reactivities for the other four trichothecenes were: 10.7% (DON), 6.1% (NIV), 2.4% (15-Ac-DON), and 0.1% (FX). Since most analysts prefer to analyse for DON rather than 3-Ac-DON, it is helpful to express the cross-reactivity relative to DON. Setting the activity for DON as 100%, the cross-reactivities for the other four trichothecenes were: 930% (3-Ac-DON), 57.4% (NIV), 22.9% (15-Ac-DON), and 0.9% (FX). Additionally, two mycotoxins (T-2 tetraol and Tri-Ac-DON) showed slight binding to the antibody when present at very high concentrations (greater than 10 000 ng ml^{-1}), where effects of the solvents (present at high levels to keep these toxins soluble) were expected to begin contributing to the response. Twelve other mycotoxins, including T-2 toxin, HT-2 toxin, T2-triol, diacetoxyscirpenol, trichothecin, iso-trichodermin, 8-hydroxy-isotrichodermin, neosolaniol, verrucarol, verrucarin A, roridin A, and sambucinol, were unreactive at concentrations at, or below, 10 000 ng ml^{-1}.

Several characteristics of the Mab were able to be inferred from the cross-reactivity data. The first was the importance of the presence of an acetate group at C-3 for the best binding. Second, the presence of a carbonyl at C-8 was required for binding, as toxins that contained either a hydroxyl at this position (T-2 tetraol, 8-hydroxy-isotrichodermin), an acetate (neosolaniol), an isovalerate (T-2, T-2 triol, HT-2), or a hydrogen (isotrichodermin, diacetoxyscirpenol, verrucarol) were all non-

reactive. Third, while binding was better for a hydroxyl group at C-15, the binding site was capable of accommodating toxins with an acetate at this position (namely, 15-Ac-DON). Fourth, while a methylene group at position 4 (i.e. as in DON) was the preferred congener for binding, the antibody binding site readily accepted a hydroxyl group (as in NIV) and, to a lesser extent, an acetate group (as in FX) at this position. Lastly, the inability of the macrocyclic trichothecenes verrucarin A and roridin A to react in the CI-ELISAs, suggests that attachment of a long chain between the C-4 and C-15 positions precludes binding to the antibody. The results show that while the antibody is most specific for 3-Ac-DON, the binding site can accommodate related molecules with slight modifications to this structure at positions 3, 4, or 15.

The cross-reactivity of the Mab will likely be an important consideration for quantitation of trichothecenes in naturally contaminated samples, particularly if 3-Ac-DON is present. While surveillance data for 3-Ac-DON is not as readily available as surveillance data for DON, several reports have indicated that 3-Ac-DON is found worldwide, although the relative contribution of this congener to trichothecene levels in cereals varies widely. The RIVM report (Pronk et al. 2002) indicated that 128 of the 545 cereal grains tested worldwide were positive for 3-Ac-DON (range 0.005–1.9 $\mu g\,g^{-1}$), while of 33 samples obtained from Europe, only one was positive (at 0.05 $\mu g\,g^{-1}$). Data from Germany reported by Schollenberger (1999, 2005) indicated that in 1998, 4% of cereal-based foods tested contained 3-Ac-DON (a mean contamination level of 0.017 $\mu g\,g^{-1}$), while in samples collected in 2000–01, the prevalence rate was only 1% in cereal-based foods. The situation may be different in parts of Asia. In Korea, seven of 39 barley samples and none of the 46 maize samples contained 3-Ac-DON (a mean contamination level of 0.065 $\mu g\,g^{-1}$; Kim et al. 1993). In addition, in Korea, two of 35 samples of healthy maize and 12 of 36 samples of mouldy maize were found to contain 3-Ac-DON (a mean contamination level of 0.2 $\mu g\,g^{-1}$; Sohn et al. 1999). In Japan, 34 samples of wheat and barley that had previously been found to contain trichothecenes were tested for the presence of acetylated derivatives of DON and NIV (Yoshizawa and Jin 1995). Of these, 25 contained 3-Ac-DON.

Since the initial development of an antibody for DON (Casale et al. 1988), several antibodies that recognize DON or related trichothecenes have been produced (Mills et al. 1990; Usleber et al. 1991; Nicol et al. 1993; Niessen et al. 1993; Sinha et al. 1995; Lee et al. 1997; Maragos and McCormick 2000; Schneider et al. 2000). In most cases, the investigators tested for cross-reactivity to NIV, although the mechanism for reporting the cross-reactivity varied. Most authors reported the data as the percentage cross-reactivity rate relative to DON (100%). Three reports showed less than a 1% cross-reactivity rate with NIV (Mills et al. 1990; Niessen et al. 1993; Maragos and McCormick 2000) and three reports showed a cross-reactivity between less than 3.0 and 6.3% (Abouzied et al. 1991; Usleber et al. 1991; Lee et al. 1997). In an additional paper, the percentage cross-reactivity rate was not given, but it could be calculated from the IC_{50}'s to be approximately 0.7% (Nicol et al. 1993). By comparison, the Mab 1-6.2.6 had a cross-reactivity rate towards NIV of 57.4% relative to DON, which is substantially higher than the previously reported antibodies.

The usefulness of a NIV antibody will depend upon the sensitivity toward the toxin as well as the cross-reactivity. In the previous literature, many of the reports do not directly state the IC_{50} for NIV, but they do generally either give a lower limit for the IC_{50}, or provide sufficient information that the IC_{50} can be roughly calculated from the reported cross-reactivity data. With one exception (Usleber et al. 1991), the estimated IC_{50}'s ranged from greater than 4500 ng NIV ml^{-1} to greater than 50 000 ng ml^{-1}. The exception was a report of a polyclonal antiserum with a modest cross-reactivity to NIV (6.3%), but with a high sensitivity for DON (IC_{50} less than 10 ng ml^{-1}), indicating the antiserum in that report may have an IC_{50} for NIV in the range 50–100 ng ml^{-1} (Usleber et al. 1991). The results for the IC_{50} of Mab 1-6.2.6 in the CI-ELISA (27.5 ng ml^{-1}) indicate that this antibody is likely to be the most sensitive antibody that has been reported for NIV.

The cross-reactivity of the antibody may also provide some insights into the nature of the immunogen (NIV–Gly–OVA). It can be speculated that the reason for the greatest response of the antibody to 3-Ac-DON was that the glycine (and, therefore, OVA) was attached through the C-3 hydroxyl position of NIV. The ester group present at the C-3 of 3-Ac-DON closely resembles the carbamate linkage that would occur if glycine were linked to NIV at this position. Administering such a conjugate to mice might be expected to yield antibodies most specific for 3-Ac-NIV, but also likely reactive with 3-Ac-DON. If the glycine were linked to the NIV at the C-15 position, the antibody might be expected to be more sensitive to 15-Ac-NIV or 15-Ac-DON than to 3-Ac-DON. Similarly, if the glycine were linked to the NIV at the C-4 position, the antibody might be expected to be more sensitive to FX. Given the observed strong cross-reactivity of Mab 1-6.2.6 with 3-Ac-DON and

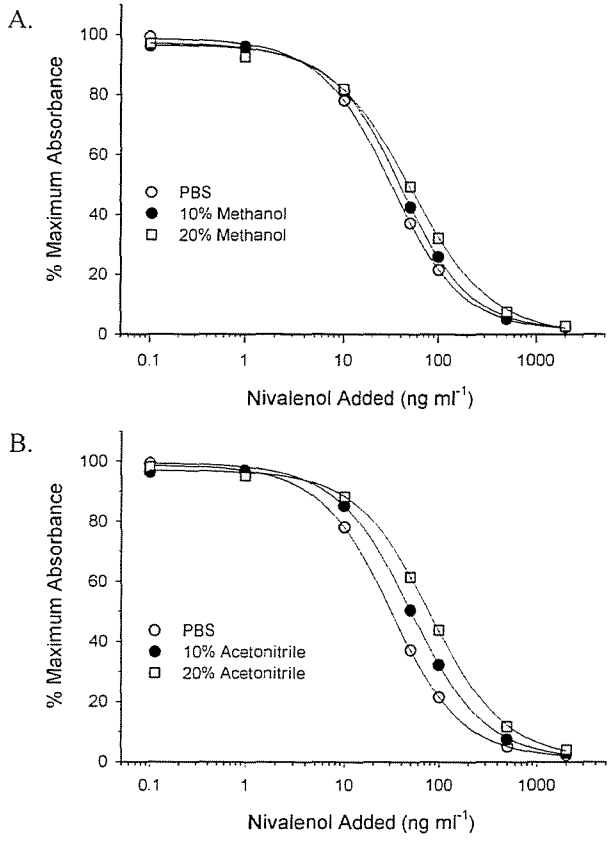

Figure 4. Effects of methanol and acetonitrile upon the CI-ELISA for nivalenol. Curves are the fit of a logistic dose-response equation to 7 concentrations of toxin and a non-toxin control. Points shown represent the average of data from either 8 plates (PBS) or 4 plates (methanol or acetonitrile levels), with quadruplicate replicates at each concentration on each plate.

relatively poorer cross-reactivities with 15-Ac-DON and FX, it can be suspected that the site of the linkage was the C-3 position. The question might be settled by determining the site of the glycine attachment to the NIV in the NIV–Gly. However, we did not intend to pursue this aspect further, since it did not affect the performance of the antibody we obtained.

Effects of solvent strength

The relative affinity of the antibody for the toxin–protein conjugate and for the toxins can also be influenced by solvent strength. We examined the effects of up to 20% methanol or 20% acetonitrile upon the CI-ELISA with NIV (Figure 4). The IC_{50}'s for NIV in the five solutions tested were: 31.1 ng ml^{-1} in PBS, 38.6 ng ml^{-1} in 10% methanol, 47.8 ng ml^{-1} in 20% methanol, 50.2 ng ml^{-1} in 10% acetonitrile, and 78 ng ml^{-1} in 20% acetonitrile. Even the low levels of methanol and acetonitrile affected assay performance, as measured by the IC_{50}. Clearly, acetonitrile had a stronger effect upon the assay than methanol, with 10% acetonitrile essentially having the same effect as 20% methanol. These results suggest that the Mab 1-6.2.6 can tolerate modest solvent strengths and may be useful in ELISAs when samples are present in up to 20% methanol or acetonitrile. However, assay sensitivity can be expected to decrease with increased solvent strength.

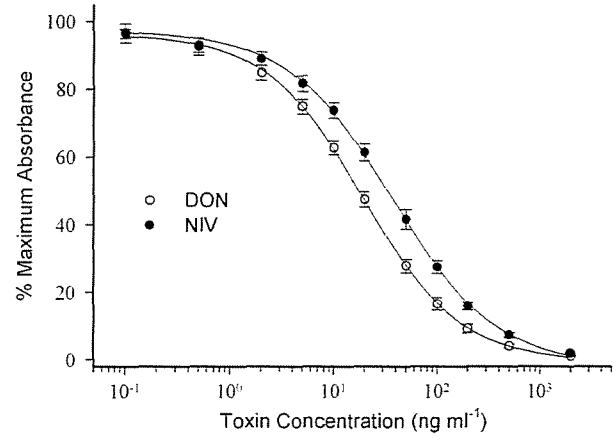

Figure 5. Response of the CD-ELISA with Mab 1-6.2.6. Curves are the fit of a logistic dose response equation to 11 concentrations of toxin and a non-toxin control. Points shown represent the average ±1 standard deviation of data from 9 replicate plates, with quadruplicate replicates at each concentration on each plate.

Competitive direct ELISAs for DON and NIV

Most of the commercial mycotoxin ELISAs use the 'competitive-direct' immunoassay format, wherein the toxin-specific antibody is coated onto the microtitre plates and the toxin (from the sample) competes with a toxin-labelled enzyme for binding to the antibody. This format has the advantage of eliminating the need for a secondary antibody and the incubation and washing steps associated with it. Given this consideration, and the ability of the Mab 1-6.2.6 to bind DON, this antibody was tested with a DON–HRP conjugate in a CD-ELISA format. The results (Figure 5) suggest that the antibody works well in this format. The IC_{50}'s for DON (16.5 ng ml^{-1}) and NIV (33.4 ng ml^{-1}) in this format compare favourably with the IC_{50}'s for DON and NIV in the CI-ELISA format (15.8 and 27.5 ng ml^{-1}, respectively). In a practical sense, the sensitivities in the two formats are essentially equivalent. Given this result, we will be investigating the use of the CD-ELISA for naturally contaminated wheat and maize.

Given the sensitivity of both the CI-ELISA and CD-ELISAs for DON and NIV, it is anticipated that

the Mab 1-6.2.6 will be a useful tool for detecting both of these mycotoxins in grains. The use of an antibody that has a cross-reactivity to both DON and NIV has advantages and disadvantages. An advantage is the ability to screen for both mycotoxins simultaneously. A disadvantage is that for positive samples, it will not be known which toxin is responsible for the positive result without a further confirmatory assay by a different technology, such as HPLC. The greatest utility of an antibody such as 1-6.2.6, which binds well both DON and NIV, may be in immunoaffinity columns, which can be used to isolate both toxins simultaneously as a clean-up before analysis by a chromatographic method such as HPLC.

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